



Original article

Reduction of nitrosative stress by methane: Neuroprotection through xanthine oxidoreductase inhibition in a rat model of mesenteric ischemia-reperfusion



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ABSTRACT

Our aim was to characterize the main components of the nitrosative response with quantitative changes of the nitrergic myenteric neurons in adjacent intestinal segments after transient superior mesenteric artery occlusion. We also tested the hypothesis that exogenous methane may modulate the evolution of nitrooxidation by influencing xanthine oxidoreductase (XOR) activity.

The microcirculatory consequences of a 50 min ischemia or ischemia-reperfusion were investigated in anesthetized rats ($n = 124$) inhaling normoxic air with or without 2.2% methane. XOR activities, nitrogen monoxide (NO), nitrite/nitrate (NO_x), and nitrotyrosine levels were measured, together with relative nitrergic neuron ratios from duodenum, ileum and colon samples. The effects of methane on XOR were also examined *in vitro*.

The intramural flow stopped only in the ileum during ischemia. The highest baseline XOR activity was found in the duodenum, which increased further during ischemia. NO and nitrotyrosine levels rose, and the nNOS-immunopositive neuron ratio and NO_x level both dropped. Reperfusion uniformly elevated XOR activity and nitrotyrosine formation, with the highest level attained in the duodenum, where the nitrergic neuron ratio remained depressed. These alterations were eliminated in methane-treated animals, XOR activity and nitrotyrosine formation decreased in all sites, and the duodenal nitrergic neuron ratio was re-established. The inhibitory effect of methane on XOR-linked nitrate reductase activity was also demonstrated *in vitro*.

With segment-specific microcirculatory alterations, the risk for nitrosative stress is highest in transiently hypoxic tissues with high endogenous XOR activities. The XOR-inhibitory effect of methane can reduce nitrooxidation and protects the nitrergic neuron population in such conditions.

1. Introduction

The enteric nervous system (ENS) contains a variety of functionally distinct neural circuits, where nitric oxide (NO) has a key role in mediating the descending inhibitory component of peristalsis [1].

Despite its important regulatory effects during normoxia and mild hypoxia [2], NO may be directly or indirectly toxic during hypoxia-reoxygenation conditions [3]. It is well established that transient reductions of the blood flow can lead to an antigen-independent inflammatory tissue response through reactive oxygen species (ROS)

Abbreviations: EPR, electron paramagnetic resonance; ENS, enteric nervous system; GI, gastrointestinal; IR, ischemia-reperfusion; IR, ischemia-reperfusion group; I, ischemic group; CH_4 , methane; IR + M, methane-treated ischemia-reperfusion group; I + M, methane-treated ischemic group; S-I + M, methane-treated sham-operated group; S-IR + M, methane-treated sham-operated ischemia-reperfusion group; MPO, myeloperoxidase; Nnos, neuronal nitric oxide synthase; NO, nitric oxide; NO_x , nitrite/nitrate; ROS, reactive oxygen species; RBCV, red blood cell velocity; S-IR, sham-operated ischemia-reperfusion group; S-I, sham-operated ischemic group; SMA, superior mesenteric artery; XOR, xanthine oxidoreductase

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generation pathways. One of the principal additional stress metabolites is peroxynitrite (ONOO⁻) [3], a derivative of NO and superoxide radicals, which can nitrosylate the thiol groups of proteins, causing conformation changes and functional or structural membrane damage due to lipid peroxidation. Subnormal oxygen concentrations impede the neuronal NO synthase (nNOS)-derived NO formation, but the evolving tissue acidosis increases the rate of alternative enzymatic NO generation from nitrite or nitrate via nitrate reductases [4].

The impairment of nitrergic neurotransmission is involved in the pathology of several clinical conditions, including gastroparesis, hypertrophic pyloric stenosis, achalasia or ischemia-reperfusion (IR) syndromes [5,6], but the consequences and mechanistic details of the nitrosative reaction on the nNOS-immunopositive gut innervation remain unclear. While nNOS can be present in up to 50% of enteric neurons [7], the distribution is not uniform. In addition, there may be large intramural differences in close association with the diverse functions of distinct gastrointestinal (GI) regions [7,8]. The heterogeneity in vascular compensatory responses adds to the complexity because the cascade of events is initiated during the ischemic phase [5,9,10] and the interconnected changes are significantly amplified with the reintroduction of the blood flow to the previously hypoxic areas. Collectively, this suggests that it is not only the neurochemical coding, but also cell density and specific perfusion characteristics of the hypoxic gut regions can all influence and shape the outcome.

Given this background, the first aim of this study was to investigate the evolution of nitrosative stress in association with a period of ischemia along the segments of the intestinal tract. In order to quantify the main features of ischemia or reperfusion-induced changes, we designed a rat model of superior mesenteric artery (SMA) occlusion where the intramural perfusion conditions of the main anatomical regions of the GI tract could appropriately be evaluated by using *in vivo* microscopic methods.

Besides NO, the intestinal lumen contains a range of potentially bioactive gases such as carbon dioxide, hydrogen sulfide, ammonia or methane (CH₄). Of interest, exogenous CH₄ influences the small bowel transit time [11,12], and a number of recent reports have demonstrated anti-inflammatory potential for CH₄-based approaches in model experiments [13,14]. These converging research findings suggested to us that an increased CH₄ input may influence the neuronal reactions to transient ischemia. Accordingly, in the second part of the study, we will set out to investigate the effects of normoxic exogenous CH₄ on the IR-induced neuron loss in the same setup.

We also hypothesized that CH₄ administration may target a major enzymatic source of reperfusion-induced superoxide formation [15]. Xanthine oxidoreductase (XOR, EC 1.17.3.2.) is abundantly present in the GI tissues both in humans and rodents [16], and, more importantly, it can catalyze the reduction of nitrite to NO under hypoxic conditions in a pH-, nitrite-, and oxygen-dependent manner [2,17–19]. In this line, we hypothesized that an association between XOR activity and the nitrergic neuron numbers might contribute to the differences in the susceptibility of the GI tract to IR damage [20]. To this aim, we designed a research protocol to detect the endogenous XOR activity changes and established biomarkers of nitrogen-centered radical formation, in parallel with GI region-specific quantitative parameters of nitrergic neurons during a standardized ischemic or IR challenge, with or without exogenous CH₄ administrations. In the last part, we used *in vitro* assays to corroborate the *in vivo* findings and to test the hypothesis that CH₄ may directly modulate XOR and XOR-linked nitrate reductase activities.

2. Materials and methods

2.1. Animals

The experiments were performed on adult male Sprague-Dawley rats ($n = 124$, average weight 328 ± 5 g) with adherence to the NIH guidelines and the EU directive 2010/63/EU for the protection of

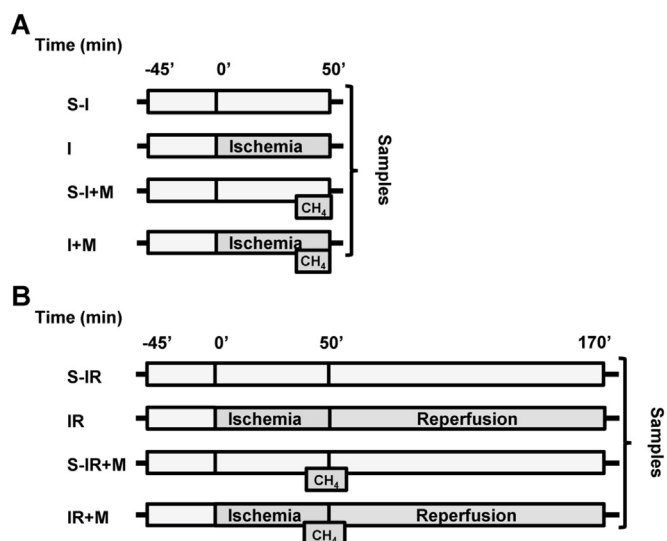


Fig. 1. Schematic picture of the experimental protocols. Four ischemic (A) and four ischemia-reperfusion groups (B) were used for the biochemical measurements and immunohistochemistry. Four additional ischemic and four additional ischemia-reperfusion groups were used for NO measurements with electron paramagnetic resonance spectroscopy.

animals used for scientific purposes. The study was approved by the National Scientific Ethical Committee on Animal Experimentation under license number V/148/2013.

2.2. Surgical procedures

The animals were anesthetized with a mixture of ketamine (45.7 mg kg^{-1}) and xylazine (9.14 mg kg^{-1} ip). For instrumentation, they were placed in a supine position on heating pads, a tracheostomy was performed and the right jugular vein was cannulated with PE50 tubing for infusion of Ringer's lactate ($10 \text{ ml kg}^{-1} \text{ h}^{-1}$ iv). The left carotid artery was cannulated for mean arterial pressure and heart rate measurements. After a sterile midline laparotomy, the SMA was dissected free and the intestine was covered with wet gauze pads. After the invasive procedures, the rats were left for 45 min to recover from the surgical stress [21].

2.3. Experimental protocols

The experiments were performed in two series (Fig. 1). In Study 1 ("ischemia alone without reperfusion"), the animals were randomly assigned to four groups: sham-operated (S-I, $n = 14$), ischemic (I, $n = 14$), CH₄-treated sham-operated (S-I + M, $n = 7$) and CH₄-treated ischemic (I + M, $n = 7$) groups (Fig. 1A). Rats in I and I + M groups were subjected to ischemia with the occlusion of the SMA distal to the major duodenal papilla for 50 min.

In Study 2 ("ischemia and reperfusion"), a second set of rats was randomly assigned to further experimental groups: sham-operated (S-IR, $n = 14$), IR ($n = 14$), CH₄-treated sham-operated (S-IR + M, $n = 7$) and CH₄-treated IR (IR + M, $n = 7$) groups (see Fig. 1B). In the IR and IR + M groups, the vascular clamp was removed from the SMA at the end of the 50 min ischemia, and reperfusion was induced and maintained for an additional 120 min.

At the end of both studies (50 min of ischemia in Study 1, and 50 min ischemia and 120 min of reperfusion in Study 2), tissue samples were collected for immunohistochemistry and biochemical analyses and then the rats were euthanized with an overdose of sodium pentobarbital (200 mg kg^{-1} iv).

2.4. CH₄ inhalation

The animals with CH₄ administration (S-I + M, I + M, S-IR + M and IR + M groups) were treated with normoxic artificial air containing 2.2% CH₄ (Linde Gas, Hungary) via a trachea cannula, as described earlier [13,21]. In Study 1 the administration of CH₄ was started during the last 10 min of ischemia, while in Study 2 it was begun 10 min before the end of ischemia and then continued for 5 min during reperfusion. The gas flow was adjusted to 300 ml min⁻¹ and regulated with a digital laboratory gas flow rotameter.

CH₄ concentrations in the ileum tissue and circulating venous blood were detected by means of photoacoustic spectroscopy as reported in accompanying studies. After a 15 min inhalation of normoxic CH₄-air mixture with a flow rate of 300 ml min⁻¹ approx. 6.6 ppm mg⁻¹ CH₄ was measured in the ileal tissue and 1.5 ppm ml⁻¹ CH₄ in the blood taken from the caval vein [21]. These data are also consistent with the previously reported values in the spinal cord after CH₄-enriched fluid injections [22].

2.5. Intravital videomicroscopy of the serosal microcirculation

The intravital orthogonal polarization spectral imaging technique [23] (Cytoscan A/R, Cytometrics, Philadelphia, PA, USA) was used to visualize the serosal microcirculation, as described earlier [24]. Quantitative assessment of the microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images. Red blood cell velocity (RBCV; $\mu\text{m s}^{-1}$) changes in the capillaries were determined in three separate fields by means of a computer-assisted image analysis system (IVM Pictron, Budapest, Hungary) [24]. Capillary perfusion rate (1/1) was determined as the length of continuously perfused microvessels per the total length of capillaries in the observational area. During quantitative assessment, we used a diameter limitation (less than 20 μm) to determine the microvascular network. Measurements of capillary blood flow were taken after the surgical procedure, then 50 min after SMA occlusion and, finally, 120 min after the release of SMA blockade.

2.6. Preparation of intestinal samples

Tissue segments were dissected from the duodenum (next to the pylorus), ileum (10 cm proximal to the ileo-cecal junction) and colon (1 cm distal to the ileo-cecal junction). In each case, the proximal part of the segment (approx. 7 cm) was used for immunohistochemical studies and the distal (2–3 cm long) portion was used for biochemical analyses. The biopsy samples were homogenized and centrifuged at 4 °C for 20 min at 24,000g.

2.7. Determination of tissue myeloperoxidase (MPO) activity

The tissue activity of MPO as an indicator of neutrophil granulocyte infiltration was measured at 37 °C in the pellet of the homogenate as described earlier [21]. The resulting data were referred to the protein content of the sample.

2.8. XOR activity

Duodenum, ileum and colon homogenates were centrifuged and XOR activity was measured in the ultrafiltered supernatant with a fluorometric kinetic assay on the basis of the conversion of pterin to isoxanthopterin in the presence (total XOR) or absence (xanthine oxidase activity) of the electron acceptor methylene blue [25]. XOR activity was calculated and expressed in $\mu\text{mol min}^{-1}\text{ mg protein}^{-1}$.

2.9. Tissue nitrite/nitrate level

Biological samples (tissue and reaction mixture in the in vitro study)

were assayed for nitrite and nitrate (NO_x) levels with the Griess reaction [26]. This assay depends on the enzymatic reduction of nitrate to nitrite, which is then converted into a colored azo compound detected spectrophotometrically at 540 nm. Briefly, the samples were divided in two (200 μl each), and the nitrite concentration was measured in the first aliquot by adding the Griess reagent (550 μl) to the samples. In parallel, 200 μl samples were mixed with 0.1 M potassium phosphate buffer (pH 7.5 buffer; 300 μl), β -NADPH (1 mM; 20 μl), FAD (0.3 μM ; 10 μl) and nitrate reductase (EC 1.7.1.1; 10 mU; Sigma-Aldrich, USA) and then incubated for 30 min at 37 °C. The Griess reagent (550 μl) was added to the mixture and incubated for 10 min at room temperature. The absorbances measured were converted to nitrite content or total NO_x content using NaNO₂ or NaNO₃ standard curves, respectively. Total NO_x was calculated and expressed in $\mu\text{mol mg}^{-1}$ protein in the case of tissue samples.

2.10. Tissue nitrotyrosine level

Free nitrotyrosine as a marker of peroxynitrite generation was measured by enzyme-linked immunosorbent assay (Cayman Chemical; Ann Arbor, MI, USA). Tissue samples were homogenized and then centrifuged at 4 °C for 10 min at 15,000g. The supernatants were collected and incubated overnight with anti-nitrotyrosine rabbit IgG and nitrotyrosine acetylcholinesterase tracer in precoated (mouse anti-rabbit IgG) microplates, followed by development with Ellman's reagent and measured spectrophotometrically at 405 and 420 nm. Nitrotyrosine content was normalized to the protein content of the intestinal homogenates and expressed in ng mg⁻¹ protein.

2.11. Immunohistochemistry

Whole-mounts were prepared from the proximal 7 cm of the dissected duodenum, ileum and colon, and immunostaining was performed as described earlier [27]. Briefly, parts of the whole-mounts were incubated with anti-human neuronal protein HuC/HuD developed in mice (Molecular Probes, Eugene, OR; final dilution 1:100), while other parts were incubated with anti-nNOS (Sigma, USA; final dilution 1:200) developed in rabbits. The whole-mounts were mounted on gelatine-coated slides, observed and photographed as described earlier [27]. Twenty digital frames taken from the duodenum, ileum and colon were analyzed with 200-fold magnification. The numbers of HuC/HuD-immunopositive (Fig. 2A) and nNOS-immunopositive neurons (Fig. 2B) per visual field (0.57 mm²) were counted in each section with Plexus Pattern Analysis software [28]. Data were expressed as percentage of nNOS-immunopositive neurons compared to the HuC/HuD-immunopositive neurons.

2.12. Electron paramagnetic resonance (EPR) spectroscopy measurements of NO

In a subset of experiments, four additional groups of ischemic animals (S-I, I, S-I + M, I + M; n = 5) and four additional groups of IR animals (S-IR, IR, S-IR + M, IR + M; n = 5) were studied. The rats underwent the same surgical procedure as described earlier and were used for the measurement of NO in the intestinal tissue with the method developed by Kozlov et al. [29]. Briefly, rats were injected subcutaneously with sodium diethyldithiocarbamate (Na-DETC; 500 mg kg⁻¹) and FeSO₄ (25 mg kg⁻¹, dissolved in 0.18 mM sodium citrate), 20 min before SMA occlusion, maintaining 5 min periods between the injections (DETC first, under the skin of the right upper inner thigh, and FeSO₄, under the skin of the left upper inner thigh 5 min later). The injected Na-DETC forms a water-insoluble DETC-Fe complex with the iron in the tissue compartment, which can bind and stabilize NO as a DETC-Fe-NO complex, allowing measurement of NO in the natural compartment where it was formed. DETC-Fe-NO complex has a characteristic EPR signal, which permits qualitative and quantitative analysis. Tissue

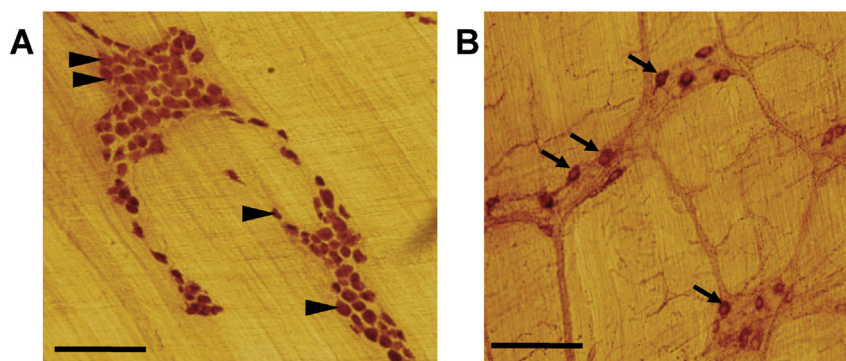


Fig. 2. Representative photograph of the ileal myenteric plexus with HuC/HuD- (A) and nNOS-immunohistochemical staining (B). Arrowheads: HuC/HuD-immunoreactive nuclei; arrows: nNOS-immunoreactive somas; scale: 250 μ m.

samples collected at baseline, after ischemia or reperfusion were shock-frozen in liquid nitrogen and stored at -80°C until measurement. EPR spectra of frozen samples were recorded at liquid nitrogen temperature with a Miniscope MS 200 EPR spectrometer (Magnetech GmbH, Berlin, Germany) with the following settings: modulation frequency 100 kHz, microwave frequency 9.429 GHz, microwave power 30 mW, modulation amplitude 6 G, spectrum range 2700–3700 G. NO levels were quantified by the amplitude of the characteristic DETC-Fe-NO signal.

2.13. In vitro study

An in vitro cell-free setup was used to examine the effects of CH_4 on XOR activity and XOR-dependent nitrosative stress. 5 ml of reaction mixture containing 10 μM sodium nitrite/nitrate (Sigma-Aldrich, USA) in XOR buffer (0.05 M KH_2PO_4 and 0.1 M EDTA; 37°C ; pH = 7.0 and pH = 7.4) was bubbled for 3 min with normoxic artificial air ($n = 6$) or normoxic air containing 2.2% CH_4 ($n = 6$) (Linde Gas, Hungary). During the initial gas saturation, a reference sample was taken from the mixture to determine the baseline NO_x level. The reaction was then started with the addition of 3 mU xanthine oxidase (Sigma-Aldrich, USA). The gas flow was adjusted to 300 ml min^{-1} and regulated with a digital laboratory gas flow rotameter. After 5 min of incubation, the samples were taken from the mixture to measure XOR activity and NO_x levels as described before. NO_x content was expressed as the difference between the initial and final concentrations in the reaction mixture ($\Delta\mu\text{M}$).

2.14. Statistical analysis

Data analysis was performed with a statistical software package (SigmaStat 12.5 for Windows, Jandel Scientific, Erkrath, Germany) and $P < 0.05$ were considered significant. The Shapiro-Wilk test was used to test normal distribution. Non-normally distributed microcirculatory data were tested with the Friedman analysis of variance on ranks, and time-dependent differences from the baseline were assessed with Dunn's method. Differences between groups were analyzed with the Mann-Whitney U test, data were displayed as median values, and the interquartile ranges of the 75th and 25th percentiles. In the case of normally distributed data, a one-way analysis of variance of repeated measures (ANOVA) was used, followed by the Holm-Sidak post hoc test for pairwise multiple comparisons in groups. These data were expressed as means $\pm$ SEM.

3. Results

3.1. Baseline hemodynamics

Baseline macrohemodynamic variables did not differ significantly in the different groups and intravital microscopy revealed homogenous

serosal perfusion in all groups under control conditions (data not shown).

3.2. Changes in serosal microcirculation during SMA occlusion

The RBCV decreased significantly in a segment-specific manner during ischemia, in the duodenum to approx. 30% of the baseline (Fig. 3A), to zero in the ileum and close to zero in the colon (Fig. 3B, C). After the release of the SMA occlusion, heterogeneous microcirculation was observed in all intestinal segments and the RBCV remained significantly lower as compared to baseline values (Fig. 3A–C). By the end of the reperfusion, the RBCV in the ileum and colon increased (Fig. 3B, C), while it remained significantly lower (approx. 25% of the baseline value) in the duodenum (Fig. 3A).

The capillary perfusion rate in the duodenum decreased to 54% of the baseline during SMA occlusion and to 18% and 16% of the baseline in the ileum and colon, respectively (Fig. 3D–F). In the ileal and colonic capillaries, the peristalsis moved the red blood cells. At the end of the reperfusion period, the duodenal perfusion rate remained significantly lower (53%) as compared to baseline values (Fig. 3D). In contrast, the perfusion rate in the ileum and colon was significantly higher than during ischemia and nearly reached baseline values by the end of observation (Fig. 3E, F).

CH_4 inhalation significantly increased the rate of perfused capillaries and the RBCV in the colon during ischemia (Fig. 3C, F) and at the end of reperfusion as well (Fig. 3C) as compared to the non-treated IR group.

3.3. Changes in biochemical parameters

The baseline characteristics of different GI segments are summarized in Table 1.

3.3.1. Tissue MPO activity

Similar MPO levels were present in the duodenal and ileal samples from sham-operated rats, while MPO activity was significantly lower in the colon, only one third of the small intestinal levels (Table 1). MPO activity did not change in the ileum during ischemia ($1.4 \pm 0.1 \text{ U mg protein}^{-1}$ vs $2.1 \pm 0.4 \text{ U mg protein}^{-1}$). However, reperfusion resulted in a significant increase in the duodenal (1.7 ± 0.3 vs $2.7 \pm 0.3 \text{ U mg protein}^{-1}$ $P < 0.05$) and ileal (1.6 ± 0.1 vs $3.3 \pm 0.3 \text{ U mg protein}^{-1}$ $P < 0.05$) samples, while significant changes were not detected in the colonic tissue after reperfusion ($0.5 \pm 0.1 \text{ U mg protein}^{-1}$ in S-IR; $0.8 \pm 0.2 \text{ U mg protein}^{-1}$ in IR).

When CH_4 inhalation was started before reperfusion, MPO activity was reduced to control levels both in the duodenum (2.7 ± 0.3 in IR vs. $1.6 \pm 0.2 \text{ U mg protein}^{-1}$ in IR+M; $P < 0.05$) and the ileum (3.3 ± 0.3 in IR vs. $1.7 \pm 0.1 \text{ U mg protein}^{-1}$ in IR+M; $P < 0.05$). There was no significant change in MPO activity in the colon after CH_4

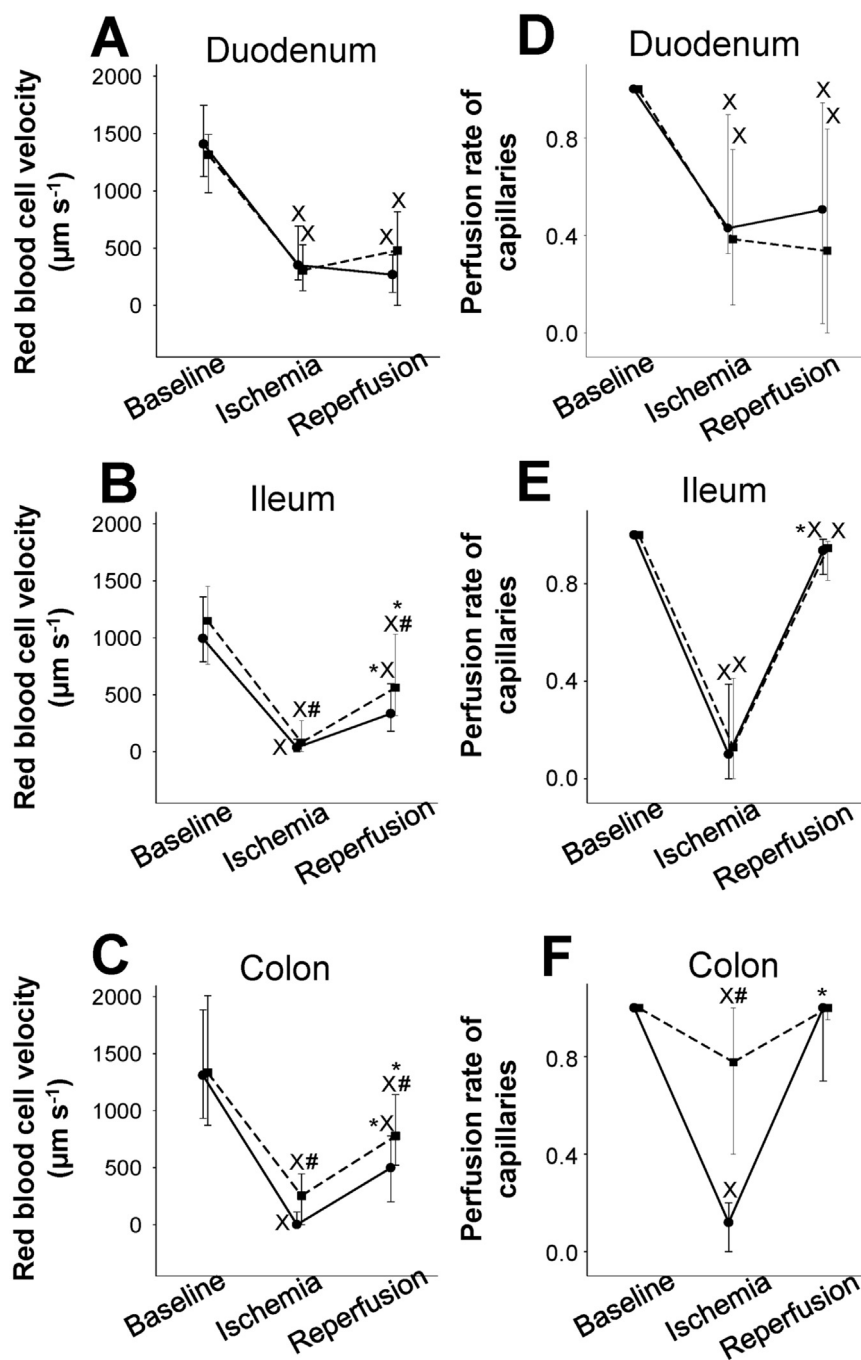


Fig. 3. Changes in red blood cell velocity (A–C) and perfusion rate (D–F) of serosal capillaries during ischemia and ischemia-reperfusion in the duodenum (A, D), ileum (B, E) and colon (C, F). Solid line – IR group; dashed line – IR + M group; medians + 25th and 75th percentiles; X $P < 0.05$ vs. baseline period, respectively, * $P < 0.05$ vs. ischemic period, respectively, and # $P < 0.05$ vs. IR.

inhalation (0.7 ± 0.1 U mg protein⁻¹ in IR + M).

3.3.2. XOR activity

XOR activity was significantly (approx. 5 times) higher in the duodenum than that in the ileum (Fig. 4A, Table 1) and significantly (approx. 20 times) higher than that in the colon (Fig. 4A, Table 1). During ischemia, there was a significant increase in XOR activity in the duodenum, but not in the ileum or colon, while reperfusion significantly increased XOR activity in all intestinal segments (Fig. 4A, B).

When CH₄ inhalation was used during ischemia, the XOR activity in the ischemic duodenum decreased to control level (Fig. 4A). The XOR activity remained reduced in the reperfused small intestinal segments and in the colon as well (Fig. 4B).

3.3.3. Tissue nitrotyrosine

Higher nitrotyrosine levels were detected in the duodenal samples from sham-operated rats than in the ileal samples, while the nitrotyrosine content was remarkably lower in the colon by approx. 65% (Fig. 5A, Table 1). During the occlusion of SMA, the nitrotyrosine levels increased significantly in the duodenum only (Fig. 5A). After reperfusion, the nitrotyrosine level was elevated significantly in the duodenum, ileum and colon as well (Fig. 5B).

With CH₄ inhalation, the nitrotyrosine content in the duodenum remained at control level during ischemia (Fig. 5A). CH₄ inhalation also reduced the nitrotyrosine content in the ischemic and reperfused small and large intestinal segments (Fig. 5B).

Table 1

Region-dependent differences in biochemical and immunohistochemical parameters in sham-operated rats.

	Duodenum	Ileum	Colon
Myeloperoxidase activity (U mg protein ⁻¹)	1.8 ± 0.2	1.4 ± 0.1	0.6 ± 0.1 [*]
Xanthine oxidoreductase activity (μmol min ⁻¹ mg protein ⁻¹)	297.7 ± 21.2 [*]	62.8 ± 4.6	13.7 ± 0.9 [*]
Nitrite/nitrate content (μmol mg protein ⁻¹)	16.4 ± 1.8 [*]	30.9 ± 2.7	36.9 ± 1.9
Nitrotyrosine content (ng mg protein ⁻¹)	6.5 ± 1.4	4.3 ± 0.4	1.5 ± 0.3
Fe-bound NO content Signal intensity (AU)	10939 ± 753 [*]	20818 ± 1406	4942 ± 481 [*]
Number of nNOS-immunopositive neurons (Neuronal number field of view ⁻¹)	26.8 ± 0.4	26.9 ± 0.4	44.5 ± 1 [*]
Proportion of nNOS-immunopositive neurons (%)	28.5 ± 1.4	24.3 ± 1	26.3 ± 1

Myeloperoxidase and xanthine oxidoreductase activity, tissue nitrite/nitrate nitrotyrosine and NO levels, number and ratio of nNOS-immunopositive neurons in the duodenum, ileum and colon in sham-operated rats. Means ± SEM.

^{*} *P* < 0.05 vs. ileum.

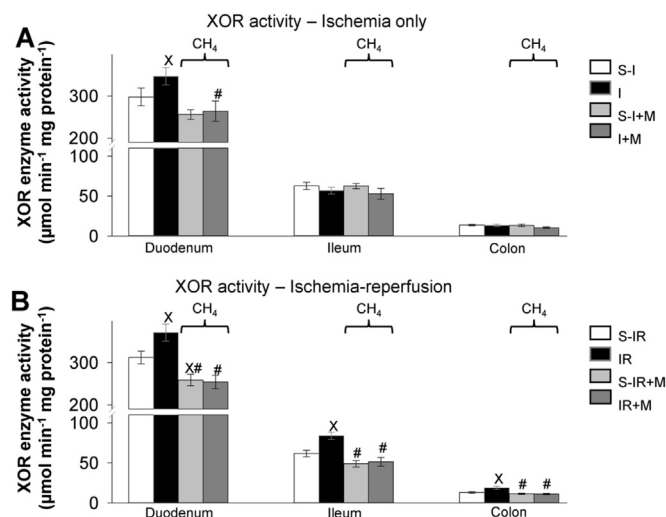


Fig. 4. Levels of tissue xanthine oxidoreductase (XOR) activity during ischemia (A) and ischemia-reperfusion (B) in the duodenum, ileum, and colon. Means ± SEM; *X P* < 0.05 vs. S-I or S-IR, respectively, and # *P* < 0.05 vs. I or IR, respectively.

3.3.4. Tissue nitrite/nitrate content

Tissue NO_x level in the duodenum was significantly lower, approx. 50% of that in the ileum and colon (Fig. 6A, Table 1). During ischemia, NO_x content decreased in the duodenum, while it significantly increased in the ileum (Fig. 6A). At the end of reperfusion, NO_x content did not change in any parts of the intestinal tract (Fig. 6B).

Inhaled CH₄ significantly increased NO_x content in the ileum during ischemia (Fig. 6A) and after reperfusion as well (Fig. 6B). It also significantly increased NO_x content in the duodenum, while this value remained unchanged in the colon (Fig. 6B).

3.3.5. Tissue NO levels

Tissue NO levels were approx. 2 times higher in the ileum than those in the duodenum, but in the colon they were only approx. 23% of those in the ileal tissue (Fig. 7A, Table 1). Mesenteric ischemia significantly increased tissue NO levels in the duodenum only (Fig. 7A),

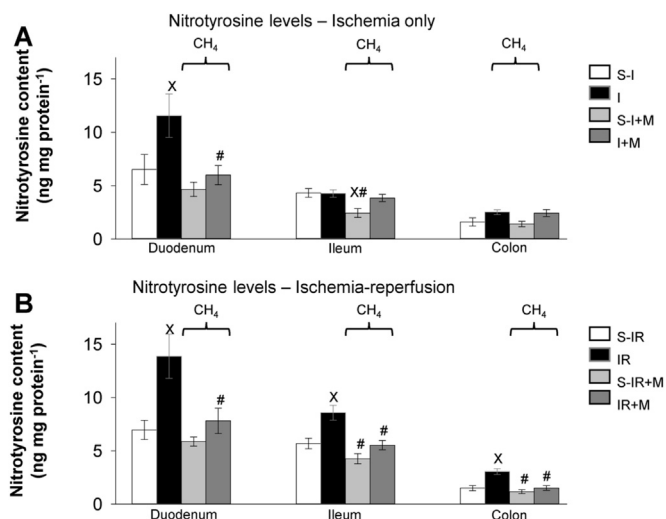


Fig. 5. Tissue nitrotyrosine content during ischemia (A) and ischemia-reperfusion (B) in separate intestinal segments. Means ± SEM; *X P* < 0.05 vs. S-I or S-IR, respectively, and # *P* < 0.05 vs. I or IR, respectively.

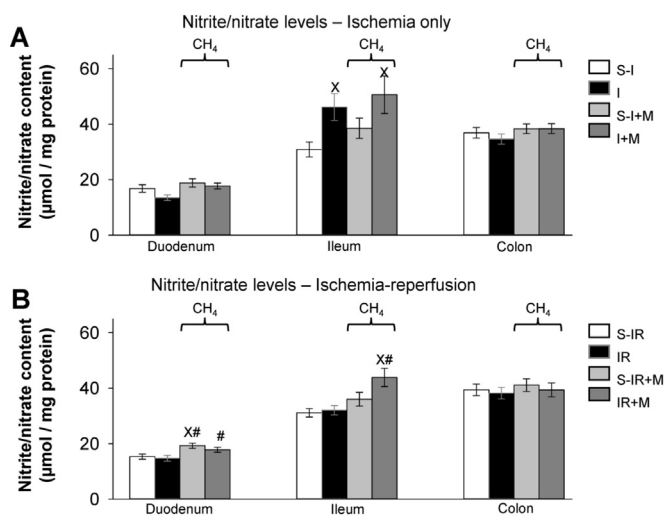


Fig. 6. Tissue nitrite/nitrate content during ischemia (A) and ischemia-reperfusion (B) in the rat duodenum, ileum, and colon. Means ± SEM; *X P* < 0.05 vs. S-I or S-IR, respectively, and # *P* < 0.05 vs. I or IR, respectively.

and at the end of the reperfusion period the tissue NO levels remained at control level in all intestinal segments (Fig. 7B).

CH₄ inhalation significantly reduced elevated duodenal NO production and significantly decreased ileal tissue NO levels during ischemia, while tissue NO levels did not change in the colon (Fig. 7A). After reperfusion, tissue NO levels remained at control levels in all intestinal segments (Fig. 7B).

3.3.6. Quantification of HuC/HuD- and nNOS-immunoreactive myenteric neurons

The HuC/HuD immunostaining was used as a pan-neuronal marker for quantitative immunohistochemistry (Fig. 2A), while nNOS immunostaining was used for specific labelling of the nitrergic neurons (Fig. 2B). The baseline densities of nNOS- and HuC/HuD-immunopositive neurons in the tissue samples from sham-operated rats (Table 1) corresponded to earlier published data [27]. The total number of myenteric neurons did not change significantly during ischemia or after IR. However, the ratio of nNOS-immunoreactive neurons decreased significantly in the ischemic duodenum and ileum, while changes were not observed in the ischemic colon (Fig. 8A). By the end

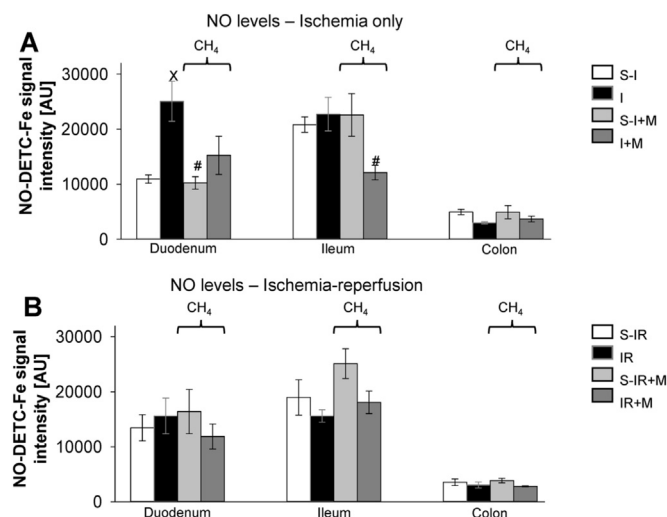


Fig. 7. Tissue NO levels during ischemia (A) and ischemia-reperfusion (B) in separate intestinal segments. The levels of tissue NO were determined by electron paramagnetic resonance spectroscopy using DETC-Fe complexes as the specific trap for NO. Means $\pm$ SEM; X $P < 0.05$ vs. S-I or S-IR, respectively, and # $P < 0.05$ vs. I or IR, respectively.

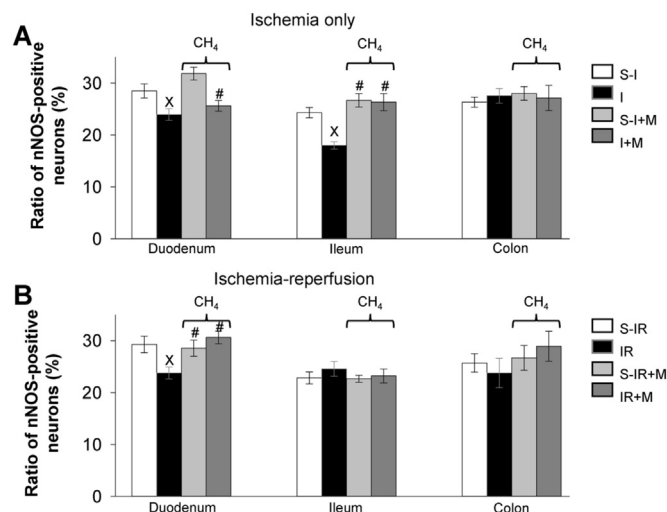


Fig. 8. Ratio of nNOS-immunopositive to HuC/HuD-immunopositive neurons in the duodenum, ileum, and colon during ischemia (A) and ischemia-reperfusion (B). Means $\pm$ SEM; X $P < 0.05$ vs. S-I or S-IR, respectively, and # $P < 0.05$ vs. I or IR, respectively.

of reperfusion, the ratio of nitrergic neurons in the duodenum remained significantly decreased, while the ratio of nitrergic neurons remained at control levels in the ileum and colon (Fig. 8B).

When CH₄ was used during the SMA occlusion, the ratio of nitrergic neurons in the duodenal samples remained decreased, while this parameter was maintained at the same level in the ileal samples from the same animals as in their respective controls (Fig. 8A). However, after reperfusion with CH₄ inhalation, the proportion of nitrergic neurons in the duodenum returned to the control level (Fig. 8B). In the colon, the proportion of nNOS-immunopositive neurons remained unchanged during SMA occlusion or after reperfusion or after CH₄ inhalation (Fig. 8A, B).

3.4. Changes in biochemical parameters in vitro

3.4.1. XOR activity

Similar to the results of the in vivo experiments, a significant decrease in XOR activity was observed after 5 min of incubation with CH₄

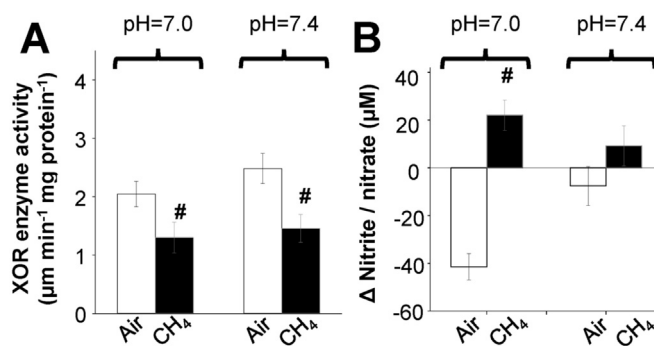


Fig. 9. Levels of xanthine oxidoreductase (XOR) enzyme activity (A) and changes in nitrite/nitrate concentration (B) of in vitro reaction mixtures of different pH after bubbling with normoxic artificial air (Air) or methane-enriched normoxic artificial air (CH₄). Means $\pm$ SEM; # $P < 0.05$ vs. normoxic artificial air.

in the reaction mixture at both pH levels (Fig. 9A).

3.4.2. Nitrite/nitrate content

The NO_x content of the reaction mixture decreased significantly at pH = 7.0 but not at pH = 7.4 during incubation with air only, reflecting the pH-dependent nitrite/nitrate reductase activity of XOR. The addition of CH₄ preserved the NO_x content of the reaction mixture (Fig. 9B).

4. Discussion

In this cross-sectional study, we characterized the distribution of nNOS-immunopositive neurons in the myenteric plexus of the rat duodenum, ileum and proximal colon to investigate the main components and consequences of a nitrosative stress response after transient intestinal ischemia. Locally available NO content is, by definition, a decisive factor in the generation of nitrogen-centered radicals. We thus detected the tissue levels of NO_x with NO and nitrotyrosine formation, and intravital microscopy was used to provide evidence for the regional microcirculatory patterns. The relative distribution of nitrergic neurons in the intestinal segments was relatively similar, but the number of nNOS-immunopositive neurons myenteric plexus increased from the duodenum towards the colon, and the tissue NO_x content showed a similar proximal to distal gradient. Since the nitrergic neuron number is directly proportional to the activity of the cellular constitutive nNOS isoform and the nNOS protein content [27], the highest level of nitrosative stress would therefore be expected in the large intestine [27].

We present three further findings, which modify this view. First, we observed significant differences in the nutritive microperfusion of the intestinal tract hit by a standard period of SMA occlusion. The intramural flow was fully stopped in the ileum during the initial ischemic episode, but the capillary perfusion did not stop completely in the duodenal and large intestinal parts, most likely due to the compensatory flow redistributions through the intact collateral networks [30]. In the duodenum, approx. 30% of the baseline blood flow was preserved during ischemia, while 5–6% was preserved in the colon during that period, thus possibly resulting in varying degrees of hypoxia and various magnitudes of reperfusion at these anatomical locations.

Second, the elevated MPO and XOR activities and the nitrotyrosine content were indicative of the presence of the nitrosative response following the hypoxia-reoxygenation cycles. However, it should be noted that the formation of nitrotyrosine was already started during the occlusion phase in the low-perfused duodenum, where the XOR activity was also significantly elevated during ischemia.

Third, we detected significant regional differences in the local NO levels, parallel to the region-specific arrangement of nitrergic myenteric neurons and XOR activity changes in the small and large intestines. The exceedingly high duodenal XOR activity decreased to a five-times lower level in the ileum and then again in the colon, reaching the lowest

values in the large intestine [20]. The tissue nitrotyrosine content was closely associated with the endogenous XOR activity; it was high in the duodenum and the lowest in the colon. The levels of NO trapped by DETC-Fe complexes in intestinal tissue, a direct marker of in-situ NO formation, changed in the same direction, high local NO content was detected in the ileum, and tissue NO levels in the colon were much lower.

The myenteric plexus between the muscle layers of the GI tract is primarily involved in the control of smooth muscle motor patterns such as peristalsis, and it has previously been demonstrated that IR insults can lead to myenteric damage with persistently reduced nNOS immunoreactivity [10]. Our results, however, provide a more complex view and suggest a dynamic change in regional nNOS-expressing myenteric neurons in close association with the GI microcirculation and the XOR-dependent arm of the nitrosative stress. What we observed was that the decrease in the ratio of nNOS-immunopositive nitrergic neurons was much higher in the ischemic ileum, a tissue completely devoid of perfusion, than in the colon, where the ratio of myenteric nitrergic neurons did not change. We also took into account that XOR can be a potent nitrite and nitrate reductase in ischemic scenarios [2,19]. It is well established that XOR is a major enzymatic source of hypoxia-induced ROS generation when both O₂ tension and the pH are transiently lowered [15,19]. In this microenvironment, the decreasing mitochondrial NADH oxidation increases the cellular NADH concentration, which in turn reduces the molybdenum cofactor site of XOR, leading to a retrograde electron flow from the FAD site. Because the affinity of xanthine substrate is dependent on the oxidized state of the molybdenum site, the reduced state is more accessible to nitrite and nitrate reduction [19]. Thus, considering the whole spectrum of nitrogen biology-related events, the local pool of NO_x, NO and nitrotyrosine production, and the degrees of flow deprivation, it is conceivable that a switch from xanthine oxidase to a dominant nitrate reductase property of XOR actively contributed to the reduction of the tissue NO_x content to generate NO in the ischemic duodenum. In contrast to the duodenal location, the XOR activity – and the nitrite/nitrate reductase capacity – of the ileum did not change during a complete flow arrest, which allows for NO_x accumulation in the non-perfused, ischemic tissues. The biological consequence of this difference is unknown, but certain data suggest that NO_x accumulation in the ischemic bowel serves a protective function during no flow conditions [31]. It has been shown that NO_x and non-enzymatically-generated NO may possess protective characteristics in hypoxic environments, as NO aids the cells in avoiding complete oxygen depletion via a reduction of the oxygen consumption of mitochondria in hypoxic cells [32,33]. This reasoning is confirmed by the available data, but direct in vivo evidence for a NADH-driven nitrite reduction of XOR requires further study.

We also demonstrated that the intestinal areas do not progress through the arterial occlusion-induced IR states at predictable rates. Reperfusion after the release of SMA occlusion caused substantial microcirculatory deterioration with prolonged capillary no-reflow and spatial flow heterogeneity in the duodenum. In the neighboring ileum, however, the intramural blood flow increased significantly, and, likewise, the colonic microcirculation was also improved by the re-established arterial circulation. In this situation, the ratio of nNOS-expressing myenteric neurons remained significantly lower in the reperfused duodenum. Meanwhile, the quantitative parameters of nNOS-expressing neurons did not change in the colon, although the cell number was the highest in this region. Additionally, there were no significant increases in the NO or NO_x levels during reperfusion in the large intestine. The reduced duodenal nitrergic neuronal ratio was associated with a more intensive nitroxidation as evidenced by significant increases in nitrotyrosine levels and XOR activity. In contrast, the XOR activity and the nitrotyrosine content were low in the reperfused colon, suggesting lower exposure of nitrergic neurons to nitrosative stress in this region. In line with this, the XOR activity and nitrotyrosine content did not change in the ischemic ileum during SMA occlusion, and the ratio of

nNOS-immunopositive neurons returned to control level after reperfusion.

Collectively, these data demonstrate the manifestation of an adaptation process in the reperfused gut in association with the degree of nitrosative stress. The responses of nitrergic ENS neurons to transient ischemia can follow similar adaptation patterns to those of their CNS counterparts; shorter periods of ischemia and reperfusion induce transient changes, but prolonged or ongoing environmental changes will pass the nNOS-expressing neurons into the second phase with permanent structural alterations and substantial neuronal loss [6,10,34]. The reactive adaptation of nitrergic neurons may follow a similar mechanism to that in the cardiomyocytes, where the translocation of nNOS content from the sarcoplasmic reticulum to the plasma and mitochondrial membranes was demonstrated [35,36].

In this protocol, we have investigated the consequences of an increased CH₄ input with special emphasis on nitrergic neuronal changes and XOR-linked reactions. CH₄ is an omnipresent, less reactive gas molecule, with a close symbiosis with other, potentially bioactive gases in the intestinal tissues. While CH₄ is conventionally believed to be physiologically inert, experimental data demonstrate that it can modulate the GI transit time [11,12]. Of note, many human studies have reported correlations between breath CH₄ levels and peristaltic functions as CH₄ production is usually associated with a constipation-related phenotype [11,12,37]. However, the relevance of these findings remain controversial, and the relationship between endogenous microbial or non-microbial CH₄ generation and specific diseases has still not been determined [38].

More importantly, many other findings demonstrated that higher concentrations of CH₄ can influence key events of inflammation [39]. It has been shown that CH₄ induces anti-inflammatory responses via master switches such as Nrf2/Keap1 or NF-κB [11–14,22,40]. Moreover, CH₄ treatment can increase Nrf2 expression and transcriptional activity in neurons, leading to an upregulated profile of antioxidant enzymes (heme oxygenase-1, superoxide dismutase, catalase and glutathione) [41]. There is also experimental evidence that CH₄ supplementation can influence the motor and sensory deficits of spinal cord ischemia and the neurologic complications of diabetic retinopathy [22,42].

In our model, the elevated XOR activity was significantly decreased in all intestinal locations with an increase in the CH₄ input; in parallel, nitrotyrosine formation was significantly suppressed. The reduced XOR activity was associated with a higher nNOS-immunopositive neuron ratio and increased NO_x pool in the duodenum. Furthermore, normoxic CH₄ administration significantly decreased tissue NO levels in the hypoxic duodenal tissue already during the ischemic phase. These phenomena were accompanied by reduced activation of an inflammatory response during reperfusion.

The best possible explanation for these events is that the remaining scant blood flow kept the underperfused, posts ischemic duodenum in hypoxic conditions and XOR retained the nitrite/nitrate reductase activity. The addition of inhibitory CH₄ thus preserved the NO_x content within the tissues. It should be added that in contrast to the CH₄-containing colonic gas environment, the gas composition of the stomach is actually quite similar to the inhaled air, and in contrast to the duodenum, the colon wall is persistently exposed to constant pro-inflammatory stimuli. Thus, it is tempting to speculate that the CH₄ is required within specific parts of the GI tract to maintain the inflammatory signals at rest.

The in vitro protocol provided further data on the inhibitory effect of CH₄-containing artificial air on the XOR-linked nitrate reductase activity, but the exact mechanism by which CH₄ modulates XOR activity remains unclear. The apolar CH₄ molecule can be dissolved in the hydrophobic nonpolar lipid tails of the phospholipid membranes, and it may influence its physicochemical condition, essential for the normal functioning of embedded proteins and ion channels. As an analogy, halothane can modify the G-protein-mediated signaling pathways and

the kinetics of acetylcholine-activated ion channels in a like manner [43]. Indeed, it has recently been shown that CH₄ directly alters the activity of transmembrane enzymes (i.e. glutathione peroxidase and superoxide dismutase) [40] and that of the phosphoinositide 3-kinase and protein kinase B pathway, thus increasing the activity of glycogen synthase kinase 3 [13,14,39]. Nevertheless, XOR is not a transmembrane protein; thus, other possibilities, such as changing the hydrophobic environment around the FAD site, should be taken into account to understand the in vitro mechanism of CH₄ action [44–46].

5. Conclusions

In the present study, a histological analysis of the nitrergic neuronal networks was conducted simultaneously with the detection of relevant intestinal biochemical changes and serosal microcirculatory alterations as physiological endpoints, and it was possible to quantify the consequences of transitory ischemia exactly. We have found evidence for large differences in the parameters of intestinal microperfusion and the tissue XOR activities in response to a standard SMA occlusion. Specifically, the perfusion differences led to region-dependent progress in nitrosative stress elements and likewise to differences in the reactions of nitrergic neurons in the myenteric plexus. In this scenario, the risk for nitrosative stress is highest in the duodenum and lowest in the large intestine despite the fact that the colon contains the highest NO_x levels and the largest number of nNOS-immunopositive cells.

In addition, we provided new evidence that inhaled CH₄ may influence XOR activity and nitrogen biology. Whereas there is a growing scientific rationale for the use of CH₄ supplementation, there is still no evidence that CH₄ in the endogenously produced concentration range plays similar roles. It is also unknown whether the findings in this model are applicable to other conditions, and clarification of the exact mechanisms of action requires further in-depth investigations. However, the data support the notion that exogenous CH₄ is neuroprotective by influencing XOR activity and its related effects on the pathways involved in the nitrosative stress response in the rat GI tract.

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Conflict of interest

All authors have no potential financial or ethical conflicts of interest regarding the contents of the submission.

Authors contributions

M. Z. Poles, J. Kaszaki and M. Boros conducted the study and drafted the manuscript. Data were collected and assessed by M. Z. Poles, N. Bódi, M. Bagyánszki, A. T. Mészáros, G. Varga, S. Szűcs, A. Nászai and L. Kiss. Analysis and interpretation of EPR data was done by A. V. Kozlov and A. T. Mészáros. Critical revision of the manuscript for important intellectual content was performed by M. Boros and É. Fekete. M. Boros, J. Kaszaki supervised the study. All authors approved the final version of the manuscript.

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